

Accepted Manuscript

Methylation of the Oxytocin Receptor Gene in Clinically Depressed Patients Compared to Controls: the Role of OXTR rs53576 Genotype

Dr. I. Reiner, M.H. Van IJzendoorn, M.J. Bakermans-Kranenburg, S. Bleich, M. Beutel, H. Frieling



PII: S0022-3956(15)00081-3

DOI: [10.1016/j.jpsychires.2015.03.012](https://doi.org/10.1016/j.jpsychires.2015.03.012)

Reference: PIAT 2591

To appear in: *Journal of Psychiatric Research*

Received Date: 15 January 2015

Revised Date: 16 March 2015

Accepted Date: 17 March 2015

Please cite this article as: Reiner I, Van IJzendoorn M, Bakermans-Kranenburg M, Bleich S, Beutel M, Frieling H, Methylation of the Oxytocin Receptor Gene in Clinically Depressed Patients Compared to Controls: the Role of OXTR rs53576 Genotype, *Journal of Psychiatric Research* (2015), doi: 10.1016/j.jpsychires.2015.03.012.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Title page**Methylation of the Oxytocin Receptor Gene in Clinically Depressed Patients Compared to Controls: the Role of OXTR rs53576 Genotype**

Reiner, I¹, Van IJzendoorn MH², Bakermans-Kranenburg MJ², Bleich, S³, Beutel, M¹,
Frieling, H³.

Affiliation:

¹ Department of Psychosomatic Medicine and Psychotherapy University Medical Center; Mainz; Germany

² Center for Child and Family Studies, Rommert Casimir Institute for Developmental Psychopathology, Leiden University, Leiden, The Netherlands

³ Molecular Neuroscience Laboratory; Department of Psychiatry, Social Psychiatry and Psychotherapy, Hannover Medical School (MHH), Hannover, Germany

RUNNING TITLE:

Methylation Oxytocin Receptor Gene Depression

Corresponding Author:

Dr. Iris Reiner

University Medical Center Mainz

Department of Psychosomatic Medicine and Psychotherapy

Untere Zahlbacher Str. 8; 55131 Mainz; Germany

iris.reiner@unimedizin-mainz.de

Phone: +49-6131-178124

Fax: +49-6131-175563

Abstract

The emerging field of epigenetics provides a biological basis for gene-environment interactions relevant to depression. We focus on DNA methylation of exon 1 and 2 of the oxytocin receptor gene (*OXTR*) promoter. The research aims of the current study were to compare *OXTR* DNA methylation of depressed patients with healthy control subjects and to investigate possible influences of the *OXTR* rs53576 genotype. The sample of the present study consisted of 43 clinically depressed women recruited from a psychosomatic inpatient unit and 42 healthy, female control subjects – mean age 30 years (SD = 9). DNA methylation profiles of the *OXTR* gene were assessed from leukocyte DNA by means of bisulfite sequencing. Depressed female patients had decreased *OXTR* exon 1 DNA methylation compared to non-depressed women. The association between depression and methylation level was moderated by *OXTR* rs53576 genotype. Exon 2 methylation was associated with *OXTR* rs53576 genotype but not with depression. Our findings suggest exon-specific methylation mechanisms. Exon 1 methylation appears to be associated with depressive phenotypes whereas exon 2 methylation is influenced by genotype. Previously reported divergent associations between *OXTR* genotype and depression might be explained by varying exon 1 methylation. In order to further understand the etiology of depression, research on the interplay between genotype, environmental influences and exon-specific methylation patterns is needed.

Keywords:

Oxytocin; methylation; depression; *OXTR*; biomarkers; exon-specific;

Introduction

With a life-time prevalence in the range of 10-15% in the general population, depression is a frequent and serious mental disorder, affecting the subjects' quality of life in domains such as psychosocial functioning, work productivity, relationship quality, and mortality risk (Lépine and Briley, 2011). In spite of extensive research on both genetic as well as environmental factors contributing to depression, the pathogenic mechanisms leading to depression are largely unknown. The emerging field of epigenetics may provide insights in the molecular pathways between genotype and the phenotype; the aim of the current study was to explore if depression is associated with different methylation patterns of the oxytocin receptor gene (*OXTR*).

Reviews of studies estimating the heritability of the depressive disorders using behavior-genetic approaches generally propose a range of .30 - .50 and suggest even higher heritability for severe and recurrent forms of depression (Levinson, 2006, Sullivan et al. , 2000). Molecular-genetic research of potential genes associated with depression mainly focused on monoamine-related genes. The serotonin-transported-linked polymorphic region (*5-HTTLPR*) in *SLC6A4*, the gene that codes for the serotonin transporter, has been of particular interest, leading to ambiguous results (Hoehe et al. , 1998, Lotrich and Pollock, 2004, Ogilvie et al. , 1996). So far, no specific genetic variant has been reliably identified as single and "direct" risk factor for depression.

Recently, much attention has been given to the neuropeptide oxytocin in research of depression-related feelings such as loneliness and distrust (Lee et al. , 2009). Oxytocin operates through the oxytocin receptor that is coded by *OXTR*, located on chromosome 3p25.3. The *OXTR* SNP rs53576 comprises a guanine (G) to adenine (A) substitution and has been studied widely in relation to attachment and social behavior. Several studies, mostly

using an A-allele dominant genetic model, indicate that the A-allele is a “risk allele” for reduced levels of empathy and prosociality: Carriers of at least one A-allele have been found to be at higher risk for autism (Wu et al. , 2005), mothers with AA- and AG-genotypes portrayed lower maternal sensitivity than homozygote G-allele carriers (Bakermans-Kranenburg and van Ijzendoorn, 2008). Also, A-allele homozygotes (AA) were found to be lonelier (Lucht et al. , 2009). In contrast, homozygote G-allele carriers (GG) were found to be more optimistic and (Saphire-Bernstein et al. , 2011), and to have better emotional regulation abilities (Rodrigues et al. , 2009). Other studies, however, report no association between the *OXTR* rs53576 G-allele and prosocial effects (Cornelis et al. , 2012, Kawamura et al. , 2010) (see for a meta-analysis Bakermans-Kranenburg and van Ijzendoorn, 2014). Little is known about the influence of *OXTR* rs53576 on major depression: two studies reported no significant relationship between *OXTR* rs53576 genotypes and childhood-onset mood disorders (Strauss et al. , 2010) or treatment outcome in depressed patients (Mendlewicz et al. , 2012). Studies including environmental factors suggested that homozygote G-allele (GG) carriers may be at higher risk for depression (Costa et al. , 2009).

Environmental influences may confer a risk to depression by affecting the genome epigenetically (Schroeder et al. , 2010). Epigenetics refers to the study of all changes in gene function that are not encoded by the DNA itself; therefore, epigenetic mechanisms concern chemical markers at or alongside the DNA, but not modifications of the genetic code itself. Different forms of epigenetic regulation are known, and two of them are particularly crucial for the understanding of psychiatric diseases: histone modification and DNA methylation. In histone modification, histone proteins compacting DNA strands are chemically modified at the so called “histone tail” through acetylation, phosphorylation, methylation or other posttranslational modifications, which may change the accessibility of the DNA strand (Rodenhiser and Mann, 2006). DNA methylation refers the methylation of Cytosine residues

within a CpG dinucleotide. Formerly it was believed that DNA methylation mainly occurred in CpG dense regions of the genome (CpG islands) that are often associated with the gene promoter region. Today it is known that DNA methylation can also occur outside of CpG islands and throughout the genome, and that intronic CpG methylation or CpG methylation at CpG island shores are also of functional relevance. The former view that DNA methylation within the promoter region leads to gene silencing, has also been challenged recently, as more and more reports also show a positive relation between methylation and expression (Ehrlich and Lacey, 2013). Lately, the interaction between genotypes and DNA methylation has also found increased attendance, as some genotypes can affect DNA methylation throughout the whole genome (Chen et al. , 2015).

DNA methylation is an “environmentally responsive” process, reflecting gene-environment interactions (Meaney, 2010), and is therefore of special interest for the pathogenesis of depression. Several animal studies highlight that early life stress may contribute to the development of depression by influencing DNA methylation of genes associated with stress regulation (Murgatroyd et al. , 2009, Weaver et al. , 2004, Weaver et al. , 2005). In humans, methylation of the *OXTR* promoter region of exon 3 was found to increase within ten minutes of psycho-social stress (Unternaehrer et al. , 2012). However, there is no agreement on how increased versus decreased methylation status in different gene segments is associated with *OXTR* expression. In mice, increased DNA methylation of the *OXTR* gene promoter was found to be associated with increased *OXTR* expression (Mamrut et al. , 2013). Particularly methylation in CpG site 7, which is located at the SP1 binding sequence, was found to be associated with increased *OXTR* expression in various brain regions (Harony-Nicolas et al. , 2014). In humans however, increased methylation of *OXTR* exon 1, 2 and 3 was linked with decreased *OXTR* expression in the temporal cortex in autistic patients vs non-autistic controls

(Gregory et al. , 2009). A reporter-gene study functionally characterizing the *OXTR* promoter showed that DNA methylation of the CpG island spanning exons 1 and 2 was associated with a decreased expression of the reporter-gene product (Kusui et al. , 2001).

Taking findings from animal and human studies together, the inhibitory effect of *OXTR* methylation on gene expression is equivocal. Moreover, methylation in different *OXTR* gene fragments and exons may lead to increased and decreased gene expression, respectively. Nonetheless, there is evidence that *OXTR* methylation a) is associated with psychiatric phenotypes in humans and, b) “guides” *OXTR* expression in brain-specific areas. Even though systematic studies on the effects of possible confounders on DNA methylation of *OXTR* are lacking, several factors are known to affect DNA methylation in general. Among those confounders are age (Steegenga et al. , 2014), body mass index (Na et al. , 2014), antidepressant use (Melas et al. , 2012), alcohol consumption (Bönsch et al. , 2004), and tobacco smoking (Hillemacher et al. , 2008, Zeilinger et al. , 2013).

To our knowledge, ours is the first study testing whether depressed patients differ from mentally healthy individuals in *OXTR* methylation levels. We expected that *OXTR* methylation would differ in clinically depressed versus healthy individuals. In order to examine possible molecular genetics influences, we also tested whether differences in methylation is moderated by *OXTR* rs53576 genotype. Due to prior findings on the effect of antidepressants, age, alcohol, body mass index (BMI) and smoking on methylation levels, these variables were included as covariates.

Methods and Materials

Sample

The study was approved by the Ethics Committee of the State Board of Physicians of Rhineland-Palatinate (Germany). All participants provided their written informed consent to participate in this study. The total sample consisted of 85 premenopausal women aged 19-52 (Mean = 30.1 years, SD = 9.0 years). 58 % (n = 49) of the participants had a high school degree, 38 % (n = 33) secondary education and 4% (n = 3) lower secondary education. All participants were Caucasians fluent in German language. The clinical sample consisted of 43 depressed patients, who were recruited from the inpatient unit of the Department of Psychosomatic Medicine and Psychotherapy, University Medical Centre Mainz. The control group consisted of 42 healthy control subjects who were recruited by flyers and posters and were matched for age and education.

Inclusion criteria for the patient group were the diagnosis of Depression and/or Dysthymia using the German version of the Structured Clinical Interview for DSM-IV Disorders; SCID-I and SCID-II; (Wittchen et al. , 1997). Patients with borderline, antisocial or narcissistic personality disorders as well as patients with eating disorders or substance abuse were not eligible. Data collection for the patient group was time of admission. In the control group, women who were currently in psychotherapy or had a mental disorder diagnosis (using SCID-I and SCID-II) were excluded from further study participation. Generally, pregnant and breastfeeding women and subjects who suffered from adrenocorticotrophic, gynecological and neurological diseases were excluded. Factors possibly affecting DNA methylation like smoking, alcohol consumption, caffeine/theine consumption, pregnancy or obstetric complications were assessed using a semi-structured interview. The patient group had the following main diagnoses: Major depression (n = 42), dysthymia (n = 1). Comorbid conditions were anxiety disorders (n = 18), dysthymia (n = 8), and depersonalization disorder (n = 4). One subject from the control group could not be genotyped and was therefore

excluded from further data analyses. A description of all variables analyzed as possible confounders is given in table 1.

Genotyping of OXTR rs53576

Genomic DNA was isolated from 200 μ L frozen human venous blood using the NucleoMag® Blood 200 μ L Kit (Macherey & Nagel, Dueren) on a Biomek® NXP Laboratory Automation Workstation (Beckman Coulter, Brea, CA). The region of interest flanking rs53576 in *OXTR* intron III (ENSG00000180914) was amplified by Touchdown PCR using HotStarTaq® Master Mix Kit (Qiagen, Hilden), primers gOXTR_rs53576_F_431 (5'-GGCCCACCATGCTCTCCACATCACTG-3') and gOXTR_rs53576_R_432 (5'-TCAGGAGCGTTGGACTGGGAGGAAGG-3') and 1 μ L gDNA (20 – 80 ng). PCR steps were performed as followed: 97°C 1 min; 95°C 30 sec, 70°C 45 sec, 72°C 60 sec (15 cycles; incr. -1°C); 95°C 30 sec, 60°C 45 sec, 72°C 60 sec (25 cycles); 72°C 5 min. The 237 bp fragment was determined by Sanger sequencing using BigDyeTerminator v3.1 Cycle Sequencing Kit (BDT v3.1) on a 3500xL genetic analyzer (both: AppliedBiosystems, Austin, TX). SNP-genotype rs53576 was analyzed using the Sequence Scanner v1.0 software (AppliedBiosystems, Austin, TX). (AppliedBiosystems, Austin, TX).

Distribution of rs53576 was: 42.9% GG, 48.8% GA, and 8.3% AA. Deviance of allele-frequencies in depressed patients and healthy controls from Hardy-Weinberg Equilibrium as well as differences of allele and genotype frequencies between the groups were tested using the online tool from the Institute for Human Genetics (Helmholtz Center Munich, Germany) available at <http://ihg.gsf.de/ihg/snps.html>. Neither depressed patients nor healthy controls showed a deviation of genotype frequencies from Hardy-Weinberg-Equilibrium (depressed patients: $F_{(1)} = -0.13$; $P = 0.41$; controls: $F_{(1)} = -0.09$; $P = 0.55$). Due to the skewed distribution

of genotypes, and in line with previous studies, we used a dominant model contrasting AA/AG vs GG genotypes (Kumsta and Heinrichs, 2013).

Methylation

Fasting blood samples for DNA extraction were drawn on admission. Genomic DNA was extracted from whole frozen EDTA-blood with the QIAamp® DNA Blood Mini Kit (QIAGEN AG, Hilden, Germany) according to the manufacturer's protocol. Afterwards, 500 ng of genomic DNA were modified by sodium-bisulfite using the EpiTect® Bisulfite Kit (QIAGEN AG, Hilden, Germany). Sodium-bisulfite deaminates cytosines in CpG dinucleotides to uracils, whereas methylated cytosines are protected from alteration.

Primers were designed to amplify a region covering three fragments (amplicon 2A, amplicon 2B, amplicon 2C) of the *OXTR* gene containing 71 CpG sites within exon 1, intron 1 and exon 2. Primer sequences as well as fragment sizes and chromosomal position are listed in *table 2*.

To amplify a specific product, a nested touchdown PCR was performed (15 cycles of 95°C for 30sec, 63°C-48°C (- 1°C per cycle) for 45sec and 72°C for 60sec ; 25 cycles of 95°C for 30sec, 48°C for 45sec and 72°C for 60sec).

Subsequently each PCR product was visualized on a standard 2.0% agarose gel, followed by purification using Agencourt AMPure XP beads (Beckman Coulter GmbH, Krefeld, Germany). Sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. A maximum concentration of 50ng purified PCR product was applied for the extension reaction. The oligonucleotides of the second PCR were used as sequencing primers. After dye-terminator removal with Agencourt CleanSEQ System (Beckman Coulter), the

products were analyzed on an Applied Biosystems® 3500xL DNA Analyzer (Applied Biosystems).

The obtained sequences were analysed using the ESME software package that determines the DNA methylation levels from the sequence trace files. ESME performs quality control, normalizes the signals, corrects for incomplete bisulfite conversion, aligns generated bisulfite sequence and reference sequence to compare C to T values (forward sequence) and G to A values (reverse sequence) peaks at CPG-sites (Lewin et al. , 2004). Only CpG sites yielding valid results in >70% of all samples were used for further analyses. In fact only 36 CpG sites within exon 1 and 7 CpG sites in exon 2 fulfilled this criterion while no CpG site within intron 1 yielded good enough measurements to be included into the analyses.

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics 20 (IBM, New York, NY). All variables were normally distributed according to the Kolmogorov-Smirnov test. Therefore, parametric methods were applied. Data are given as mean (SD). All statistical tests were two-tailed with a significance level of $\alpha = 0.05$. ~~Confidence intervals and *p* values of post hoc test were Bonferroni-corrected for multiple comparisons.~~

To analyse the differences in DNA methylation between patients and controls, linear mixed models were performed for both exons separately. In a first model, the effect of depression on OXTR methylation was checked (dependent variable: methylation; fixed factors: CpG position; depression group; interaction between CpG position and depression group to check, if effects were evenly distributed across the promoter). To assess the effect of the OXTR genotype, this variable was included as fixed factor and interactions between OXTR and depression group were included in the model. In subsequent analyses, effects of possible

confounding variables (age, BMI, smoking, alcohol consumption, antidepressant use) were tested.

Results

Exon 1: Effects of depression on OXTR methylation

The linear mixed model on methylation level with CpG position and group (patients versus controls) showed a significant main effect of CpG position ($F_{(35;2709)}=73.25$; $p < 0.001$) and a significant difference between patients and controls ($F_{(1;2709)}=16.26$; $p < 0.001$) with a decreased level of methylation in the patient group (figure 2a). As no interaction between CpG position and group was observed ($F_{(35; 2709)}=0.91$; $p = 0.624$), group specific methylation patterns seem to be evenly distributed across exon 1 (figure 2b).

Insert Figure 2 (A and B) about here

Exon 1: Genotype x depression interaction

No significant main effect of the *OXTR* genotype was found (Figure 3a). However, the interaction between group and *OXTR* genotype was significant ($F_{(1;2706)}=4.07$; $p = 0.044$). Patients homozygous for the G-allele had lower levels of methylation of exon 1 than those carrying at least one A-allele (estimated marginal means (SE): GG: 0.065(0.003) vs. AA/AG: 0.072(0.003)), whereas controls homozygous for the G-allele had higher methylation levels than those carrying at least one A-allele (EMM: GG: 0.082(0.003) vs. AA/AG: 0.077(0.003); Figure 3b).

Insert Figures 3a and 3b about here

Exon 2: Effects of OXTR rs53576 on OXTR methylation

We found, again, a significant main effect of the CpG-position ($F_{(6; 531)}=91.54$; $p < 0.001$), but no differences between patients and controls ($F_{(1;531)}=0.512$; $p = 0.475$; Figure 4a). In a second linear mixed model with *OXTR* genotype and CpG position as factors we found a significant main effect of *OXTR* genotype ($F_{(1;529)}=40.63$; $p < 0.001$; Figure 4b) and a significant interaction between CpG-position and genotype ($F_{(6;529)}=9.95$; $p < 0.001$; Figure 4c) on exon 2 methylation level. Parameter estimates revealed that this effect was mainly driven by CpG 37 and CpG 39. No significant group by genotype interaction was observed.

Confounding factors

To check for possible confounders, we tested the influence of age, BMI, current antidepressant use, alcohol use (AUDIT sum score) and smoking status (yes/no) on methylation. We found that higher BMI and smoking were associated with lower exon 1 methylation ((BMI: $F_{(1;2701)}=13.64$; $p < 0.001$; smoking: $F_{(1;2701)}=7.82$; $p = 0.005$) while exon 2 methylation increased with age ($F_{(1;528)}=4.25$; $p = 0.04$). Covarying BMI and smoking, and age respectively, the main effects of depressed vs non-depressed group (exon 1: $F_{(1;2704)}=12.55$; $p < 0.001$) and *OXTR* genotype (exon 2: $F_{(1; 532)}=35.33$; $p < 0.001$) and the group by genotype interaction (exon 1: $F_{(1;2704)}=4.99$; $p = 0.026$) remained significant.

Discussion

The research aim of our study was to compare methylation status of the oxytocin receptor gene *OXTR* in clinically depressed versus non-depressed females. Our main finding was that

exon 1 methylation was significantly decreased in depressed patients; the effect was, however, moderated by *OXTR* rs53576 genotype. G-allele homozygous patients had decreased exon 1 methylation compared to patient group A-allele carriers. In contrast, in healthy control subjects, G-allele homozygous participants had higher exon 1 methylation than A-allele carriers. Exon 2 methylation was solely influenced by genotype and was lower in G-allele homozygous individuals, independent of clinical status or other environmental factors. Exon-specific methylation patterns, e.g. regarding *5HTTLPR*, have been reported in previous studies on lymphoblast cell lines and were also observed in a clinical sample, yet, the underlying mechanisms have not been revealed (37, 38).

Our study suggests that *OXTR* exon 1 methylation in particular is associated with phenotype traits such as depression, smoking and body weight, whereas exon 2 appears to be more “biologically driven” and less sensitive to environmental factors. Exon 2 methylation might be associated with phenotypes or clinical diagnoses that are less related to socio-emotional and environmental factors, such as autism. *OXTR* genotype influences both exon 1 and exon 2 methylation and, for exon 1, interacts with depression in the prediction of methylation level. Further research is needed to understand the dynamics between genotype, exon- and CpG-position, and methylation. Our study contributes to the understanding of depression as we, for the first time, report that clinically depressed patients portray specifically decreased methylation in exon 1. This finding raises questions on the association between decreased exon 1 methylation and altered or decreased *OXTR* expression, which may contribute to disturbed processing of social stimuli as observed in depression.

Several limitations of the present study need to be acknowledged. First, the present sample consisted of adult females before menopause, thus, replication is needed in male samples and female samples after menopause. Compared to other molecular genetic studies, the sample of

this study is relatively small in size, but importantly, it consists of clinically depressed individuals and a carefully matched mentally healthy control group. Longitudinal studies are needed to more fully establish causal relationships between *OXTR* promoter region methylation and depression.

Our findings show that the relation between DNA methylation, gene expression and depression is far more complex than previously appreciated (Baker-Andresen et al. , 2013). Further research on *OXTR* methylation in depressive and other clinical groups needs to involve exon-specific analyses to contribute to a deeper understanding of the functional relevance of different exons and CpG positions. Exon-specific methylation may also impact in different ways on central and peripheral oxytocin levels and may explain contradictory results on associations between depression, *OXTR* genotypes, and plasma oxytocin levels (Cyranowski et al. , 2008, Ozsoy et al. , 2009, Parker et al. , 2010). After promising results on the “prosocial and positive emotions enhancing” effect of intranasal administered oxytocin in non-clinical samples, preliminary studies on intranasal administration with depressed samples revealed disappointing results (Mah et al. , 2013, Pincus et al. , 2010). Although the mechanisms are not fully understood, previous research suggests that the positive impact of intranasal oxytocin administration may be diminished in clinical disorders that are strongly associated with adverse environmental factors and negative socioemotional childhood experiences such as implicated in depression and posttraumatic stress disorder (Bakermans-Kranenburg and Van IJzendoorn, 2013). Based on these findings, we cautiously suggest that the inconsistent effects of the pharmacotherapeutic use of oxytocin might be biologically explained by exon-specific *OXTR* methylation. Future research on oxytocin administration should include exon-specific *OXTR* methylation status to test for a potential mediating influence.

References

- Baker-Andresen D, Ratnu VS, Bredy TW. Dynamic DNA methylation: a prime candidate for genomic metaplasticity and behavioral adaptation. *Trends in neurosciences*. 2013;36:3-13.
- Bakermans-Kranenburg M, Van IJzendoorn M. Sniffing around oxytocin: review and meta-analyses of trials in healthy and clinical groups with implications for pharmacotherapy. *Translational psychiatry*. 2013;3:e258.
- Bakermans-Kranenburg MJ, van IJzendoorn MH. Oxytocin receptor (OXTR) and serotonin transporter (5-HTT) genes associated with observed parenting. *Social cognitive and affective neuroscience*. 2008;3:128-34.
- Bakermans-Kranenburg MJ, van IJzendoorn MH. A sociability gene? Meta-analysis of oxytocin receptor genotype effects in humans. *Psychiatric genetics*. 2014;24:45-51.
- Bönsch D, Lenz B, Reulbach U, Kornhuber J, Bleich S. Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism. *Journal of neural transmission*. 2004;111:1611-6.
- Chen L, Pan H, Tuan TA, Teh AL, MacIsaac JL, Mah SM, et al. Brain-derived neurotrophic factor (BDNF) Val66Met polymorphism influences the association of the methylome with maternal anxiety and neonatal brain volumes. *Development and psychopathology*. 2015;27:137-50.
- Cornelis MC, Glymour MM, Chang SC, Tchetgen EJ, Liang L, Koenen KC, et al. Oxytocin receptor (OXTR) is not associated with optimism in the Nurses' Health Study. *Molecular psychiatry*. 2012;17:1157-9.
- Costa B, Pini S, Gabelloni P, Abelli M, Lari L, Cardini A, et al. Oxytocin receptor polymorphisms and adult attachment style in patients with depression. *Psychoneuroendocrinology*. 2009;34:1506-14.

Cyranowski JM, Hofkens TL, Frank E, Seltman H, Cai H-M, Amico JA. Evidence of dysregulated peripheral oxytocin release among depressed women. *Psychosomatic Medicine*. 2008;70:967-75.

Ehrlich M, Lacey M. DNA methylation and differentiation: silencing, upregulation and modulation of gene expression. *Epigenomics*. 2013;5:553-68.

Gregory SG, Connelly JJ, Towers AJ, Johnson J, Biscocho D, Markunas CA, et al. Genomic and epigenetic evidence for oxytocin receptor deficiency in autism. *BMC medicine*. 2009;7:62.

Harony-Nicolas H, Mamrut S, Brodsky L, Shahar-Gold H, Barki-Harrington L, Wagner S. Brain region-specific methylation in the promoter of the murine oxytocin receptor gene is involved in its expression regulation. *Psychoneuroendocrinology*. 2014;39:121-31.

Hillemacher T, Frieling H, Moskau S, Muschler MA, Semmler A, Kornhuber J, et al. Global DNA methylation is influenced by smoking behaviour. *European Neuropsychopharmacology*. 2008;18:295-8.

Hoehe MR, Wendel B, Grunewald I, Chiaroni P, Levy N, Morris-Rosendahl D, et al. Brief research communication: serotonin transporter (5-HTT) gene polymorphisms are not associated with susceptibility to mood disorders. *American journal of medical genetics*. 1998;81:1-3.

Kawamura Y, Liu X, Akiyama T, Shimada T, Otowa T, Sakai Y, et al. The association between oxytocin receptor gene (OXTR) polymorphisms and affective temperaments, as measured by TEMPS-A. *J Affect Disord*. 2010;127:31-7.

Kumsta R, Heinrichs M. Oxytocin, stress and social behavior: neurogenetics of the human oxytocin system. *Current opinion in neurobiology*. 2013;23:11-6.

- Kusui C, Kimura T, Ogita K, Nakamura H, Matsumura Y, Koyama M, et al. DNA methylation of the human oxytocin receptor gene promoter regulates tissue-specific gene suppression. *Biochemical and biophysical research communications*. 2001;289:681-6.
- Lee HJ, Macbeth AH, Pagani JH, Young WS, 3rd. Oxytocin: the great facilitator of life. *Progress in neurobiology*. 2009;88:127-51.
- Lépine J-P, Briley M. The increasing burden of depression. *Neuropsychiatr Dis Treat*. 2011;7:3-7.
- Levinson DF. The genetics of depression: a review. *Biological psychiatry*. 2006;60:84-92.
- Lewin J, Schmitt AO, Adorján P, Hildmann T, Piepenbrock C. Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplicates. *Bioinformatics*. 2004;20:3005-12.
- Lotrich FE, Pollock BG. Meta-analysis of serotonin transporter polymorphisms and affective disorders. *Psychiatric genetics*. 2004;14:121-9.
- Lucht MJ, Barnow S, Sonnenfeld C, Rosenberger A, Grabe HJ, Schroeder W, et al. Associations between the oxytocin receptor gene (OXTR) and affect, loneliness and intelligence in normal subjects. *Progress in neuro-psychopharmacology & biological psychiatry*. 2009;33:860-6.
- Mah BL, Van IJzendoorn MH, Smith R, Bakermans-Kranenburg MJ. Oxytocin in postnatally depressed mothers: its influence on mood and expressed emotion. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. 2013;40:267-72.
- Mamrut S, Harony H, Sood R, Shahar-Gold H, Gainer H, Shi Y-J, et al. DNA methylation of specific CpG sites in the promoter region regulates the transcription of the mouse oxytocin receptor. *PloS one*. 2013;8:e56869.
- Meaney MJ. Epigenetics and the biological definition of gene× environment interactions. *Child development*. 2010;81:41-79.

- Melas PA, Rogdaki M, Lennartsson A, Björk K, Qi H, Witasp A, et al. Antidepressant treatment is associated with epigenetic alterations in the promoter of P11 in a genetic model of depression. *The International Journal of Neuropsychopharmacology*. 2012;15:669-79.
- Mendlewicz J, Crisafulli C, Calati R, Kocabas NA, Massat I, Linotte S, et al. Influence of COX-2 and OXTR polymorphisms on treatment outcome in treatment resistant depression. *Neuroscience letters*. 2012;516:85-8.
- Murgatroyd C, Patchev AV, Wu Y, Micale V, Bockmühl Y, Fischer D, et al. Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nature neuroscience*. 2009;12:1559-66.
- Na YK, Hong HS, Lee DH, Lee WK, Kim DS. Effect of Body Mass Index on Global DNA Methylation in Healthy Korean Women. *Molecules and cells*. 2014.
- Ogilvie A, Battersby S, Fink G, Harmar A, Goodwin G, Bubb V, et al. Polymorphism in serotonin transporter gene associated with susceptibility to major depression. *The Lancet*. 1996;347:731-3.
- Ozsoy S, Esel E, Kula M. Serum oxytocin levels in patients with depression and the effects of gender and antidepressant treatment. *Psychiatry research*. 2009;169:249-52.
- Parker KJ, Kenna HA, Zeitzer JM, Keller J, Blasey CM, Amico JA, et al. Preliminary evidence that plasma oxytocin levels are elevated in major depression. *Psychiatry research*. 2010;178:359-62.
- Pincus D, Kose S, Arana A, Johnson K, Morgan PS, Borckardt J, et al. Inverse effects of oxytocin on attributing mental activity to others in depressed and healthy subjects: a double-blind placebo controlled fMRI study. *Frontiers in psychiatry*. 2010;1.
- Rodenhiser D, Mann M. Epigenetics and human disease: translating basic biology into clinical applications. *Canadian Medical Association Journal*. 2006;174:341-8.

- Rodrigues SM, Saslow LR, Garcia N, John OP, Keltner D. Oxytocin receptor genetic variation relates to empathy and stress reactivity in humans. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106:21437-41.
- Saphire-Bernstein S, Way BM, Kim HS, Sherman DK, Taylor SE. Oxytocin receptor gene (OXTR) is related to psychological resources. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108:15118-22.
- Schroeder M, Krebs MO, Bleich S, Frieling H. Epigenetics and depression: current challenges and new therapeutic options. *Current opinion in psychiatry*. 2010;23:588-92.
- Steggenga WT, Boekschoten MV, Lute C, Hooiveld GJ, de Groot PJ, Morris TJ, et al. Genome-wide age-related changes in DNA methylation and gene expression in human PBMCs. *AGE*. 2014:1-18.
- Strauss JS, Freeman NL, Shaikh SA, Vetro A, Kiss E, Kapornai K, et al. No association between oxytocin or prolactin gene variants and childhood-onset mood disorders. *Psychoneuroendocrinology*. 2010;35:1422-8.
- Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: review and meta-analysis. *American Journal of Psychiatry*. 2000;157:1552-62.
- Unternaehrer E, Luers P, Mill J, Dempster E, Meyer A, Staehli S, et al. Dynamic changes in DNA methylation of stress-associated genes (OXTR, BDNF) after acute psychosocial stress. *Translational psychiatry*. 2012;2:e150.
- Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. *Nature Neuroscience*. 2004;7:847-54.
- Weaver IC, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ, et al. Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. *The Journal of Neuroscience*. 2005;25:11045-54.

Wittchen HU, Zaudig M, Fydrich T. SKID. Strukturiertes Klinisches Interview für DSM-IV.

Achse I und II. Handanweisung. Göttingen: Hogrefe; 1997.

Wu S, Jia M, Ruan Y, Liu J, Guo Y, Shuang M, et al. Positive association of the oxytocin receptor gene (OXTR) with autism in the Chinese Han population. *Biological psychiatry*. 2005;58:74-7.

Zeilinger S, Kühnel B, Klopp N, Baurecht H, Kleinschmidt A, Gieger C, et al. Tobacco smoking leads to extensive genome-wide changes in DNA methylation. *PloS one*. 2013;8:e63812.

Figure Legends

Figure 1: Scheme of the OXTR gene and associated CpG island

The OXTR gene consists of four exons and a large intronic sequence between exons 1 and 2. The whole region from exon 1 to exon 3 consists of one CpG island. The figure depicts the three PCR fragments sequenced in this study covering the largest part of the CpG island and including exons 1 and 2. Dots mark the position of CpGs that were included into the analysis.

Figure 2 Difference of OXTR exon 1 methylation levels between depressed patients and healthy controls

Patients with depression exhibit reduced methylation levels of OXTR exon 1. (A) estimated marginal means [EMM] derived from mixed linear modelling show a significant lower methylation level in patients (***: $p < 0.001$; p-value of fixed effect of group (patients vs. controls). (B) mean methylation at every single CpG position of exon 1. Error bars represent standard errors [SEM].

Figure 3: Effect of OXTR rs53576 genotype on methylation levels in exon 1

(A): No differences between A-allele carriers and GG homozygous probands were found for OXTR exon 1 methylation. (B) Differences in OXTR exon 1 methylation between patients and controls are mainly driven by G-homozygous carriers of OXTR rs53576. Statistical details are summarized in the results section.

Figure 4: OXTR exon 2 methylation

(A): No significant effect of depression on OXTR exon 2 methylation occurred, while (B) A-allele carriers exhibit significantly increased methylation levels at exon 2. (C) Significant CpG x genotype interaction, genotype effect on OXTR exon 2 methylation is mainly driven by CpGs 37 and 39.

Bars represent estimated marginal means [EMM] derived from linear mixed modelling, error bars represent standard errors. Further statistical details are summarized in the results section.

ACCEPTED MANUSCRIPT

Acknowledgements

We would like to express our special thanks to all patients and control subjects for their participation in this study. We also thank the project coordinators Gotje Trojan and Linda Müller-Engling.

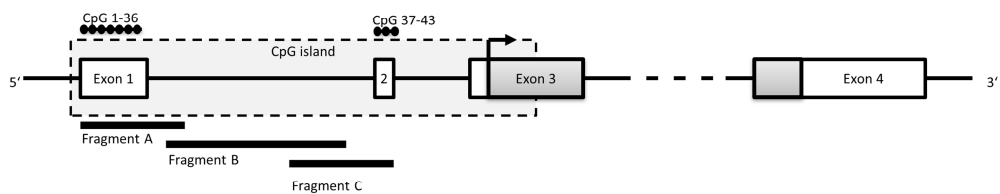
ACCEPTED MANUSCRIPT

Table 1: Description of study population

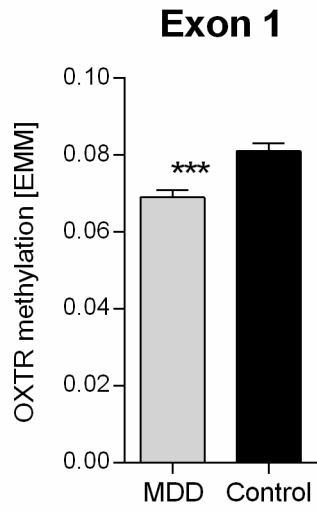
	Patients n=43				Healthy Control Women n=42				Student's T-test		
	mean	SD	min	max	mean	SD	min	max	T	df	P
Age [yrs]	29,93	8,959	19	49	30,36	9,146	20	52	-0,217	83	0,828
BMI [kg/m ²]	25,0113	12,43760	17,21	101,01	23,8447	5,06205	17,24	38,74	0,564	83	0,574
AUDIT sum score	3,44	3,534	0	18	4,14	3,324	0	18	-0,802	83	0,425
	yes	%	no	%	yes	%	no	%	Fisher's exact test		
									P		
Smoking	14	32,56	29	67,44	12	28,57	30	71,43	0,815		
Antidepressant use	14	32,56	30	67,44	0	0	42	100	<0.001		

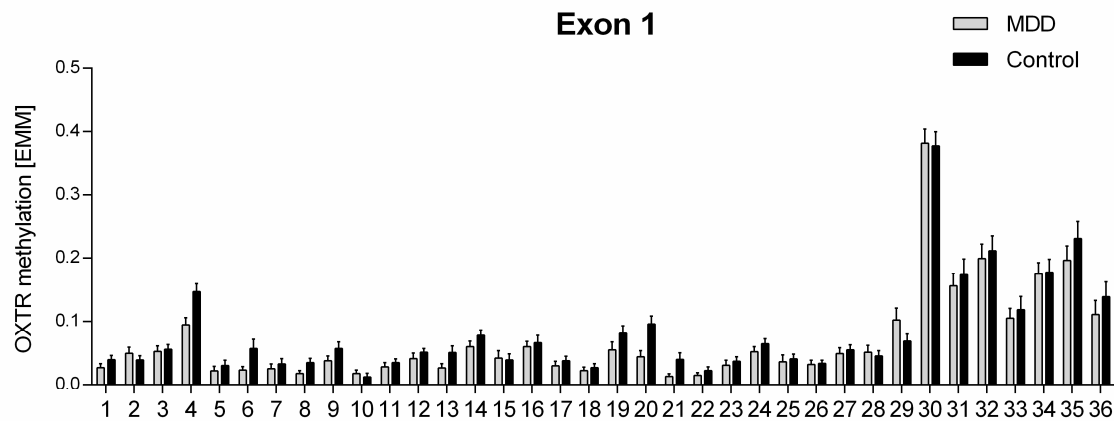
Table 2: Primers and positions of OXTR fragments

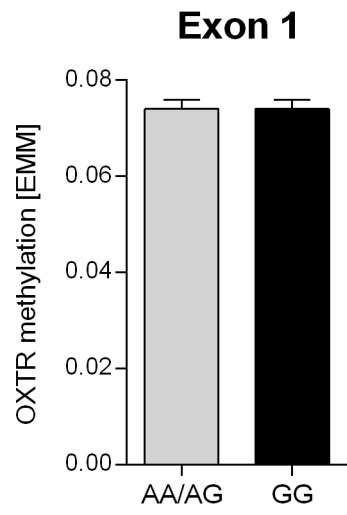
Fragment	PCR	Primer	Sequence	T _m (°C)	Position in relation to Exon 1 (bp)	Product size (bp)	Location on Chromosome 3 (alternate assembly HuRef)
A	1A	OXTR_A_F1_80	TTATTGTAATAAAATTTATTTGTTAAGG	56	-21 to +405	427	>gi 157731950 ref AC_000135.1 :8744960-8745386
		OXTR_A_R2_83	CCCCTACTTAACACCCAACCTACCT	65			
	2A	OXTR_A_F2_82	AATTTATTTGTTAAGGTTTTGGGA	55	-8 to +400	409	
		OXTR_A_R1_81	ACTTAACACCCAACCTACCTACACC	64			
B	1B	OXTR_B_F1_88	GGTGTAGGTAGTTGGGTGTTAAG	63	+376 to +694	318	>gi 157731950 ref AC_000135.1 :8744671-8744988
		OXTR_B_R2_91	CTACTAAAAAAACCCCTACCTCAA	60			
	2B	OXTR_B_F2_90	GTAGGTAGTTGGGTGTTAAGTAGGG	66	+379 to +682	303	
		OXTR_B_R1_89	CCCCTACCTCAAAACCCAAAATAC	64			
C	1C	OXTR_C_F1_84	GGGGAAGGATTAGAGTTGGGGTTT	65	+780 to +1205	425	>gi 157731950 ref AC_000135.1 :8744160-8744584
		OXTR_C_R2_87	AAAAACCCAACCTCATCTAAAACA	56			
	2C	OXTR_C_F2_86	TTAGAGTTGGGGTTTTTAGGTAA	58	+789 to +1198	409	
		OXTR_C_R1_85	CAACTCATCTAAAACAACAAAACACAACC	65			

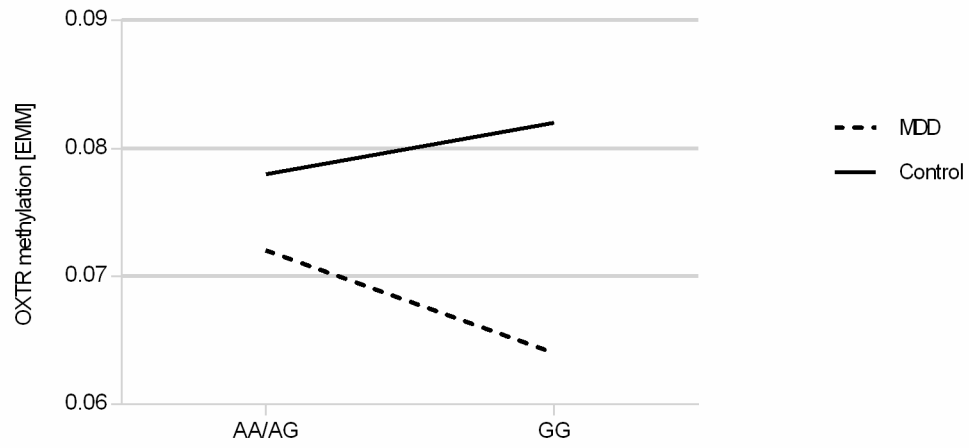


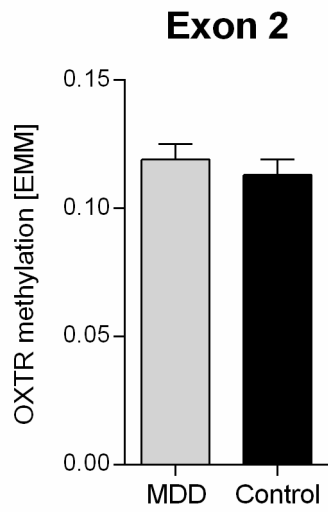
ACCEPTED MANUSCRIPT

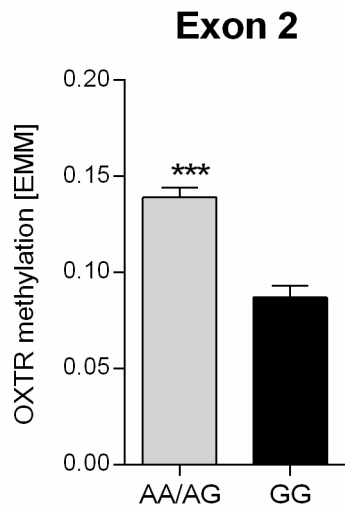




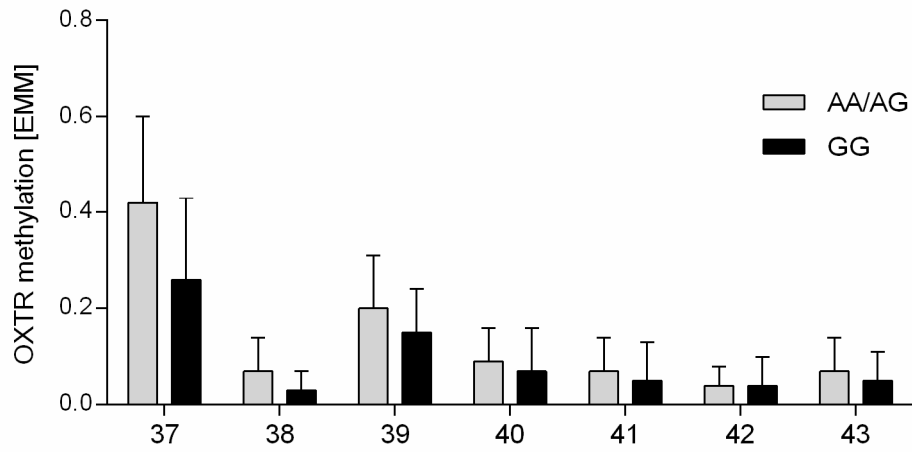








Exon 2



Highlights

- We study DNA OXTR methylation in clinically depressed patients
- We report exon-specific methylation patterns of OXTR
- Depression is associated with decreased OXTR exon 1 methylation
- OXTRrs53576 genotype is associated with OXTR exon 2 methylation
- OXTRrs53576 genotype and methylation patterns are interacting

Contributors

IR and HF designed the study; IR conducted data collection and the main literature search and wrote the first draft of the paper. HF was responsible for lab experiments and statistical analyses. IR, MI, MB and HF developed the conceptual idea of the paper. MI and MB provided critical feedback on the manuscript, suggested additional analyses and critical revisions, and edited the manuscript for clarity and precision. SB and MB contributed to the data analyses and interpretation and assisted with text editing. All authors read and approved the final version of the manuscript.

Role of the funding source

This research was supported by MAIFOR, a grant from the University Medical Mainz and the Koehler-Foundation Germany. Study sponsors were not involved in any aspect of study design; collection, analysis, or interpretation of data; preparation of the manuscript; or in the decision to submit the manuscript for publication.